TITLE OF THE INVENTION

The characterization of hupB gene encoding histone like protein of Mycobacterium tuberculosis.

FIELD OF THE INVENTION

This invention relates to the characterization of *hupB* gene encoding histone like protein of *Mycobacterium tuberculosis*. A method for differentiating *M. tuberculosis* and *M. bovis* based on the *hupB* gene .

BACKGROUND OF THE INVENTION

Numerous techniques are in vogue to differentiate between members of the MTB complex. Several researchers have demonstrated that the use of *IS6110* as a target for PCR amplification gives the best sensitivity and specificity in the diagnosis of tuberculosis. However when tested with standard mycobacterial species and strains obtained from ATCC and mycobacterial cultures isolated from clinical specimens, the target was limited in its ability to distinguish between *M. tuberculosis* complex from other mycobacteria.

Spoligotyping based detection of non-repetitive spacer sequences located between small repetitive units in the DR locus of the MTB complex strains, other genetic markers and biochemical tests have been used to differentiate between *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and *M. canetti*, (Niemann et al., 2000). Besides spoligotyping, *mtp40* gene sequence (Liebana et al., 1996),

pncA gene point mutation at position 169 (Scropio & Zhang, 1996), polymorphism of the *oxyR* locus (Sreevatsan et al., 1996), have been reported as useful targets for identification of the members of the TB complex.

Ideally, the target for PCR based detection should be that it discriminates not only among mycobacterial species but also is able to distinguish between closely related members of the MTB complex.

Although PCR techniques have been widely evaluated in the diagnosis of tuberculosis, the reports have generally focused on the detection of *M. tuberculosis*. However their success has been limited to differentiating between the tuberculosis complex and non-tuberculous mycobacteria.

Here we report a PCR assay that enables the precise identification of closely related mycobacteria belonging to the MTB complex. *hupB* gene encoding histone-like protein of *M. tuberculosis* has been exploited as a target for detection and differentiation of *M. tuberculosis* and *M. bovis*. The *hupB* gene target not only permits differentiation of *M. tuberculosis* from *M. bovis*, but also from among other members of the MTB complex, non-tuberculous mycobacteria as well as non-mycobacterial species tested. The assay would prove useful especially in developing countries with a high incidence of infected livestock (Grange, 2001). *M. bovis* has been known to spread to humans from infected cattle by the aerosol route or by consumption of infected / contaminated dairy products (Zoonotic tuberculosis) (Moda et al., 1996; Cosivi et al., 1998). Although bovine tuberculosis had been largely eradicated in developed countries, recently resurgence of bovine tuberculosis has been reported (www.defra.gov.uk/animalh) and continues to occur in developing countries. The epidemiological impact of bovine tuberculosis on human health has not been assessed and is a major lacuna in developing countries. However

with reports of tuberculosis due to *M. bovis* in AIDS patients (Bouvet et al., 1993; O'Reilly et al., 1995) and with increasing incidence of tuberculosis globally, rapid and reliable diagnostic assays are required not only for detection but also identification of the pathogenic mycobacteria in clinical samples. This is essential for prompt diagnosis, treatment and control of tuberculosis. Identification of human pathogenic mycobacteria becomes all the more relevant with the need to develop alternate new generation vaccines for human use.

Immunogenecity of HupB protein: Two methods were used to identify mycobacterial constituents associated with human response namely the T cell blot and immuno – subtraction assays (Prabhakar et al., 1998). The 30kDa fraction of the mycobacterial lysate was found to induce the highest lymphoproliferative index among the tuberculin reactors. In immuno-subtractive assays a prominent reactive band was similarly seen at approximately 30kDa. The 30kDa protein was electro-eluted from the SDS-PAGE gel and purified to homogeneity.

Using the internal peptide sequence, Seq ID No. 8 (VKPTSVPAFRPGAQFK) a 100% identity was obtained with 16 amino acids of the cosmid CY349. The corresponding gene was later annotated and designated as the *hupB* gene (Rv 2986c, Cole et al., 1998). The protein was found to be localized in the cytoplasm and on the cytoplasmic surface of the mycobacterial membrane, by immuno-gold electron microscopy. The *hupB* gene has been classified among the DNA binding (histone like) proteins of *M. tuberculosis* (Cole et al., 1998). Primers were designed to amplify the *hupB* gene. A 645 bp amplicon was obtained in case of *M. tuberculosis*. The $\alpha^{32}P$ labeled PCR amplicon was used in Southern hybridization to establish the size, prevalence and organization of the *hupB* gene in members of the MTB complex (*M. tuberculosis* and *M. bovis*) and other mycobacterial species.

Drawbacks in the existing state of art.

Although PCR techniques have been widely evaluated in the diagnosis of tuberculosis, the reports have generally focused on the detection of *M. tuberculosis*. However their success has been limited to differentiating between the tuberculosis complex and non-tuberculous mycobacteria. Single-step PCR procedures to differentiate *M. bovis* from *M. tuberculosis* using IS6110 alone or in association with *mtp40* gene have yielded discrepant results. Further it has been shown that *mpt40* is not present in all *M. tuberculosis* strains and hence may not be useful for differentiating *M. tuberculosis* and *M. bovis* strains . The *sen X3-regX3* intergenic region (IR) has been proposed as a target sequence for differentiating members of the MTB complex from other mycobacteria. However there are limitations in the use of this target region as it cannot identify members of the MTB complex, though BCG could be discerned from related strains . In the present study the *hupB* gene target has been shown to be a target, which permits differentiation of *M. tuberculosis* from *M. bovis* and from among other members of the TB complex, non-tuberculous mycobacterial and non-mycobacterial species tested. The assay would prove useful especially in developing countries with a high incidence of infected livestock (Grange, 2001). The epidemiological impact of bovine tuberculosis on human health has not been assessed and is a major lacuna in developing countries. However with reports of tuberculosis due to *M. bovis* in AIDS patients (Bouvet *et al.*, 1993; O'Reilly *et al.*, 1995) and with increasing incidence of tuberculosis globally, rapid and reliable diagnostic assays are required not only for detection but also identification of the pathogenic mycobacteria in clinical samples. This is essential for prompt diagnosis, treatment and control of tuberculosis. Here we report a PCR, RFLP and NESTED PCR assay that enables the precise identification of closely related mycobacteria belonging to the MTB complex.

OBJECTS OF THE INVENTION

An object of this invention was :

(1) To characterize *hupB* gene encoding histone like protein of *M. tuberculosis*.

(2) To characterize mycobacterial gene as new target for novel anti-mycobacterial chemotheraputic agents.

(3) To differentiate between *M. tuberculosis* and *M. bovis* based on RFLP of the PCR generated amplicon of the *hup B* gene.

(4) To differentiate between *M. tuberculosis* and *M. bovis* based on the sequence of the PCR amplicon of the *hup B* gene .

(5) To differentiate between *M. tuberculosis* and *M. bovis* based on the nested PCR of the *hup B* gene .

SUMMARY OF THE INVENTION

PCR, PCR-RFLP : The present invention relates to a process for differentiating Mycobacterial species using primers specific to the target of *hupB* gene encoding histone like protein. A single primer pair enables amplification of the target sequence from both *Mycobacterium tuberculosis* and *Mycobacterium bovis*. The size of the amplified product can differentiate *Mycobacterium tuberculosis* from *Mycobacterium bovis* and from related and unrelated species. DNA probes that hybridize to the amplified region distinguish *Mycobacterium tuberculosis* from *Mycobacterium bovis* and from related and unrelated species. Further there is provided a process (RFLP) for differentiating of the *hupB* gene of *M.tuberculosis* and *M. bovis* by restriction digestion of PCR amplified fragments.

NESTED PCR ASSAY : The utility of taking advantage of the *hupB* gene as a target is important from the point of developing molecular biological techniques

to distinguish between members of the *Mycobacterium tuberculosis* complex. Owing to the current technical hurdles in identification of *Mycobacterium tuberculosis* and *M. bovis*, factual data on the prevalence of human or animal disease due to *M. bovis* is absent / limited. Such technology would help in establishing and documenting the authentic scenario of the extent of disease caused by *M. bovis* in live stock. The proposed NESTED PCR assay would be a major contribution to present day laborious techniques used to distinguish between *Mycobacterium tuberculosis* and *M. bovis*. In India compulsory elimination of tuberculin reactive animals cannot be practiced universally for religious and socio-economic reasons. This assay would be an asset to epidemiology programs and in the identification of infected animals. The infected animals can then be segregated thereby limiting the spread of disease. Further with the extensive availability of dairy products (meat & milk) the NESTED PCR assay described here would help in alleviating the current problem of identification of human mycobacterial pathogens. These pathogens are members of the MTB complex and are genetically similar.

FIGURE LEGENDS:

Fig: 1 Position of the *hupB* gene and Primers used to generate PCR fragments.

Panel A: The position of the primers in the *hupB* sequence, which were used in order to obtain the PCR fragments have been depicted. Primer pairs **N** (Seq ID No. 1) & **S** (Seq ID No. 2) specific for the *hupB* gene; Internal primer **M** (Seq ID No. 3) & **S** (Seq ID No. 2) specific for the C terminal part of the *hupB* gene.

Panel B, C and D : The ethidium bromide stained amplification fragments of *M. tuberculosis* and *M. bovis* generated using primer pairs **N** (Seq ID No. 1) & **S** (Seq ID No. 2) (Panel **B**), **M** (Seq ID No. 3) & **S** (Seq ID No. 2) (Panel **C**) and **F**(Seq ID No. 4) & **R** (Seq ID No. 1) (Panel **D**) were electrophoresed on polyacrylamide gels. The 645 and 618 bp (Panel **B**); 318 and 291 bp (Panel **C**); 116 and 89 bp (Panel **D**); fragments have been indicated . Lanes 1 & 4, 645 bp, 6 & 10, 318 bp , and 13, 116 bp of the of *hupB* gene / C terminal part of the gene amplification fragment obtained in *M. tuberculosis* H37Rv ; lanes 2 & 5, 618 bp of *hupB* gene, 7 & 9, 291 bp and 11,12,15-17, 89 bp of the *hupB* gene / C terminal part of the gene amplification fragment obtained in *M bovis* AN5 ; 3, 8 & 14, 100 bp molecular weight markers.

Fig: 2 Specificity analysis of $hupB_{Mt}$ based PCR assay

Amplification fragments were electrophoresed on agarose gels. Their ethidium bromide staining (Panel A & Panel B) and hybridization profiles have been shown in Panel A' & Panel B' respectively. The 645 bp probe was used (generated by PCR using **N** (Seq ID No.1) and **S** (Seq ID No.2) primers and *M. tuberculosis*, DNA.).The 645 bp fragment has been indicated. **Panels A & A'**; Lanes 1 *M. tuberculosis* H37 Rv ; 2, *M. tuberculosis* H37Ra ; 3, *M. bovis* BCG ; 4, *M. microti* ; 5, *M. xenopi* ; 6, *M. fortuitum* ; 7, *M. phlei* ; 8, *M. gordonae* ; 9, *M. vaccae* ; 10, *M. kansasii* ; 11, 100 bp Marker ; 12 , *M. intracellulare* ; 13, *M. avium* ; 14, *M. scrofulaceum* ; 15, *M. smegmatis* ; 16, *M. tuberculosis* P8497 ; 17, *M. tuberculosis* C1084 ; 18, *M. tuberculosis* 779634 ; 19, *M. chelonei* ; 20, *M. tuberculosis* P8473 ; 21, *M. gastri.*

Panel B & B' Lanes 1, *M. tuberculosis* 1207; 2, *E. coli*; 3, *N. asteroides* ; 4, *S. aureus* ; 5, *P. aeruginosa* ; 6, *S. faecalis* ; 7, *S. aureus* ; 8, *A. niger* ; 9, *A. fumigatus* ; 10, *C. albicans* ; 11, 100 bp marker ; 12, *M. tuberculosis* Erdman ; 13, *K. pneumoniae* ; 14, *M. leprae* ; 15, *M. africanum* ; 16, Negative control. Hybridisation in panels B & B' was carried out with 645 bp fragment (*Pst*I & *Nco*I digest from the plasmid pHLPMT) .

Fig: 3 Sensitivity of detection of *M. tuberculosis* DNA by *hupB* based PCR assay.

Amplification reactions were performed with serial dilutions of *M. tuberculosis* DNA (1ng to 1 fg). The ethidium bromide and hybridisation patterns are seen in panels A and B respectively. The 645 bp fragment has been indicated. Lanes 1, 1 ng ; 2, 500pg ; 3, 50 pg ; 4, 5pg ; 5, 1 pg ; 6, 500 fg ; 7, 100 fg ; 8, 50 fg ; 9, 10 fg ; 10, 5 fg ; 11, 2 fg ; 12, 1 fg ; 13, Negative control ; 14, positive control (*M. tuberculosis*) ; M, λ DNA *Hind*III digest. The detection limit was 50 pg by ethidium bromide staining and 500 fg for hybridisation.

Fig: 4 RFLP analysis of the 646 and 318bp PCR fragments.

Panel A depicts the schematic representation of the position of the primers in the *hupB* sequence, which were used in order to obtain the 645 bp and 318 bp PCR fragments. Ethidium bromide staining for 645 bp **(Panel B)** and 318 bp **(Panel C)** amplification fragments are shown. Lanes 1, *M. tuberculosis* H37Rv ; 2, *M. tuberculosis* H37Ra ; 3, *M. tuberculosis* Erdman ; 4, *M. bovis* AN5 ; 5, *M. bovis* BCG (Japan) ; 6, *M.bovis* BCG (Copenhagen) ; 7, *M. bovis* IC 378 ; 8, *M. bovis* IC 379; 9,

M. bovis IC 380 ; 10, *M. bovis* IC 381 ; 11, *M. bovis* IC 382 ; 12, PCR molecular weight marker. **Panel D**, RFLP poly- acrylamide gel analysis of 645 bp amplicon digested with *Hpa*II (lanes 1- 3) and *Hae*III (lanes 6-9) : Lane 1, *M. tuberculosis* H37Rv ; 2, *M. tuberculosis* H37Ra ; 3, *M. bovis* BCG ; 4, Negative control; M, 100 bp Molecular weight marker ; 5, *M. tuberculosis* H37Rv; 6, *M. tuberculosis* H37Ra; 7, *M bovis* BCG; 8, *M bovis* AN5.

Fig: 5 Nucleotide sequence alignment of *hupB* gene of *M. tuberculosis* and *M. bovis*:

The nucleotide sequence of the C-terminal region (326-676 bp) of *hupB* gene of standard strains of *M. tuberculosis* and *M. bovis* and clinical isolates of *M. bovis* has been aligned using GCG software. A deletion of 27 bp was seen in *hupB* sequence of all *M. bovis* strains. The 9 deleted amino acids (KAATKAPAR) between 385 to 411bp with respect to *M. tuberculosis* are shown in single letter code on the first line. Numbers in brackets refer to nucleotide position in *hupB* (*Rv2986c*). The *M.bovis* strain numbers are given on the left.

Fig: 6 Nested PCR Profile of *M. tuberculosis* and *M. bovis* Standard and Cattle derived Isolates :

The nested PCR amplified fragments of the mycobacterial strains were electrophoresed on native 8% polyacrylamide gel, shown in Lanes1 negative control; 2 molecular markers; 3 *M. tuberculosis* (H37Rv); 3 Cattle isolate identified as *M. tuberculosis*; 4 Cattle isolate identified as *M. bovis*; *M. bovis* (ICC380); and 5 *M. tuberculosis* (JALMA, Agra, Isolate).

DESCRIPTION OF THE INVENTION

A method for differentiating of the *hupB* gene of *M. tuberculosis* and *M. bovis*. The size variability of the *hupB* gene was determined using 3 sets of primers (Fig: 1, Table II) :

An embodiment the present invention provides for oligonucleotide primers which are specific amplification of the *hupB* gene of *Mycobacterium* species selected from the group consisting of Seq ID No. 1, Seq ID No. 2, Seq ID No. 3, Seq ID No. 4, Seq ID No. 5.

Another embodiment is a method for differentiating *Mycobacterium* species based on target gene encoding for histone like proteins such as *hup B* .

Yet another embodiment is wherein *Mycobacterium tuberculosis*, and or *Mycobacterium bovis* species is selected from a group of genetically related *Mycobacteria* and from unrelated microorganisms.

Another embodiment is a method wherein the pair of oligonucleotide primers comprising of Seq ID No. 1 and Seq ID No. 2; Seq ID No. 3 and Seq ID No. 2; Seq ID No. 4 and Seq ID No. 5, wherein the amplified fragments are detected by ethidium bromide staining or DNA probe hybridization.

Another embodiment is a differentiating method comprising of designing primers Seq ID No. 1, Seq ID No. 2, Seq ID No. 3, Seq ID No. 4, Seq ID No. 5, to amplify a part of the said *hup B* gene from *Mycobacterium tuberculosis* and *Mycobacterium bovis*. Amplifying a part of the target gene encoding for histone like proteins such as *hup B* of *Mycobacterium* species using said DNA as a template in a polymerase chain reaction with a pair of oligonucleotide primers.

Analyzing and validating the size of the amplified fragments. Determining the complete sequence of the said amplified fragments. Inferring from the sequence whether it is *M. tuberculosis* or *M. bovis.*

Another embodiment is a method wherein the DNA probe consists of sequence ID No. 6 or sequence ID No. 7 or a complement thereof tagged with a detectable label.

Another embodiment is a method wherein the step of differentiation consists in determining the smaller size of the amplified fragment obtained from *Mycobacterium bovis.*

Another embodiment is a method wherein the PCR amplified fragment in *Mycobacterium bovis* was 618 bp.

Another embodiment is a method wherein the PCR amplified fragment in *Mycobacterium tuberculosis* was 645 bp.

Another embodiment is a method wherein the PCR amplified fragment in *Mycobacterium bovis* was 291 bp.

Another embodiment is a method wherein the PCR amplified fragment in *Mycobacterium tuberculosis* was 318 bp.

Another embodiment is a method wherein the PCR amplified fragment in *Mycobacterium bovis* was 89 bp

Another embodiment is a method wherein the PCR amplified fragment in *Mycobacterium tuberculosis* was 116bp.

Another embodiment is a method wherein the PCR amplified fragment in *Mycobacterium bovis* was 27 bp smaller than that of *Mycobacterium tuberculosis*

Another embodiment is a method wherein differentiating *M. tuberculosis* and *M. bovis* comprising the steps of amplifying a part of the target *hup B* gene from *M. tuberculosis* and *M. bovis* in a polymerase chain reaction with primers Seq. ID No.1 and Seq. ID No.2 .Restricting the amplified fragment with *Hpa II* restriction enzyme to produce restricted fragments. Separating the restricted fragments by electrophoresis on 12% polyacrylamide gel and detecting the restricted fragments by staining with ethidium bromide.

Another embodiment is a method wherein the restricted fragment in *M. tuberculosis* was 280 bp and 150 bp.

Another embodiment is a method wherein the restricted fragment in M. bovis was 253 bp and 150 bp.

Another embodiment is *Hup B* gene (Seq ID No. 8) substantially as herein described a process as in preceding embodiments has been substantially described.

Another embodiment is *Hup B* gene (Seq ID No. 7) substantially as herein described a process as in preceding embodiments has been substantially described.

The method for differentiating *Mycobacterium* species amplifying a part of the *hup B* target gene encoding for histone like proteins of *Mycobacterium* species using said DNA as a template in a polymerase chain reaction with a pair of oligonucleotide primers. Detecting said amplified fragment of the *hup B* gene to detect the presence of *Mycobacterial* species or not and differentiating *Mycobacterium tuberculosis* from *Mycobacterium bovis* based on the size of the amplified fragment.

Oligonucleotide primers for specific amplification of the *hupB* gene of *Mycobacterium* species selected from the group consisting of Seq ID No. 1, Seq ID No. 2, Seq ID No. 3, Seq ID No. 4, Seq ID No. 5. A method for differentiating *Mycobacterium* species based on target *hup B* gene. Using DNA from culture or from clinical samples as a template in a polymerase chain reaction. Detecting the said amplified fragment of the *hup B* gene to determine whether *Mycobacterium* species is present and differentiating *Mycobacterium tuberculosis* from *Mycobacterium bovis* based on the size of the amplified fragment.

1) ***hupB* gene target DNA** Primers **N, Seq ID No. 1** (5' ggagggttgggatgaacaaagcag 3') and **S, Seq ID No. 2** (5' gtatccgtgtgtcttgacctatttg 3') were used to amplify *hupB* gene sequences. The expected size of the amplicon was 645bp in case of *M. tuberculosis*, and 618 bp in case of *M. bovis* respectively.

PCR-RFLP: A method for differentiating *M. tuberculosis* and *M. bovis*. Comprising the steps of amplifying the target *hup B* gene from *M. tuberculosis* and *M. bovis* in a polymerase chain reaction with primer pair Seq. ID No.1 and Seq. ID No.2 / Seq. ID No.3 and Seq. ID No.2 . Restricting the amplified fragment with *Hpa II* restriction enzyme to produce restricted fragments. Separating the restricted fragments by electrophoresis on 12% polyacrylamide gel

2) **The C- terminal portion of the gene**: was amplified by using :

(I) internal primer **M, Seq ID No. 3** (5' gcagccaagaaggtagcgaa 3') with **S, Seq ID No. 2** (5' gtatccgtgtgtcttgacctatttg 3'), the expected amplicon was ~ 318 bp, (Fig: 1).
The expected size of the amplicon was 318 bp in case of *M. tuberculosis*, and 291 bp in case of *M. bovis* respectively.

Nested PCR: A method for differentiating *M. tuberculosis* and *M. bovis*. Comprising the steps of amplifying a part of the target hup B gene from *M. tuberculosis* and *M. bovis* in a polymerase chain reaction. The PCR fragment obtained with primers Seq. ID No.1-N and Seq. ID No.2-S was used as target DNA in nested PCR. The C- terminal portion of the gene was also amplified by using Seq.ID. No.4-F and Seq.ID. No.5-R the expected amplicon was ~ 116 bp in case of *M.tuberculosis* and 89 bp in case of *M.bovis*,

(II) using primers **F, Seq ID No. 4** (5' ccaagaaggcgacaaagg3') with **R, , Seq ID No.** 5 (5' gacagctttcttggcggg3'). The expected size of the amplicon was 116 bp in case of *M. tuberculosis*, and 89 bp in case of *M. bovis* respectively.

Sequencing of PCR amplified fragments: Analyzing and validating the size of the amplified fragments of the *hup B* gene by determining the complete sequence of the amplified fragments. Inferring from the sequence whether it is *M. tuberculosis* or *M. bovis*. The step of differentiation consists in determining the smaller size of the amplified fragment obtained from *Mycobacterium bovis*. The PCR amplified fragment obtained using primers Sequence ID No.1 and 2 in case of *Mycobacterium bovis* was 618 bp. The PCR amplified fragment in *Mycobacterium tuberculosis* was 645 bp. Whereas the PCR amplified fragment

obtained using primers Sequence ID No.3 and 2 in *Mycobacterium bovis* was 291 bp and 318 bp in case of *Mycobacterium tuberculosis*. The PCR amplified fragment obtained using primers Sequence ID No.4 and 5 in *Mycobacterium bovis* was 89 bp. and 116bp. in *Mycobacterium tuberculosis* respectively. The PCR amplified fragment in *Mycobacterium bovis* was 27 bp smaller than that of *Mycobacterium tuberculosis* .

Mycobacterial DNA extracted from *M. tuberculosis* and *M. bovis* were used. PCR amplified fragment was obtained in both *M. tuberculosis* and *M. bovis*. However the amplicon obtained in case of *M. bovis* was slightly smaller than that obtained in case of *M. tuberculosis*. This difference was confirmed by analyzing over 50 *M. tuberculosis* and *M. bovis* strains collected from diverse sources (Table I). The DNA extracted from 3 standard strains and 4 clinical isolates of *M. tuberculosis* and *M. bovis* (BCG) were included for amplification using the *hupB* primers (**N, Seq ID No. 1** and **S, Seq ID No. 2** , / **M, Seq ID No. 3** and **S, Seq ID No. 2**, Table II). The difference in the size of amplicons obtained in case of *M. tuberculosis* and *M. bovis* was validated by RFLP (Fig: 4D) and confirmed by sequencing of the PCR fragments, (Fig: 5). The PCR fragments of the two mycobacteria were digested with *HaeIII* and *HpaII* and analyzed on 12% non-denaturing gel. Digestion of the 645 bp fragment with *HpaII* revealed that a ~ 250 bp fragment was seen in case of *M. bovis* compared to the band of ~ 280 bp size obtained in case of *M. tuberculosis*, (Fig: 4D). Analyzing the sequence of the PCR fragments showed that in *M. bovis* there was a deletion of 27 bp corresponding to 9 amino acids, (Fig: 5). As a result of this deletion the PCR amplicon obtained in case of *M. bovis* was 618 bp, 27 bp smaller than the PCR fragment obtained in case of *M. tuberculosis* (645 bp), (Fig: 4 B,C).

Results, obtained with the amplicon generated in the C - terminal portion of the gene using **M** and **S** primers on digestion with *Hpa*II, showed differences matching to the differences seen in case of the PCR fragment obtained using the *hupB* primers (**Seq ID No. 1- N** and **Seq ID No. 2- S**) indicating that the PCR-RFLP assay utilizing either the PCR fragment obtained using the *hupB* primers (**N, Seq ID No. 1- N** and **S, Seq ID No. 2- S**) / the C terminal primers (**Seq ID No. 3- M** and **S, Seq ID No. 2- S**) did distinguish between *M. tuberculosis* and *M. bovis*.

The utility of the *hupB* gene as a target in diagnosis and identification pathogenic mycobacteria in bovine tuberculosis has been demonstrated, (Table IV-VII). The sensitivity and specificity of the assay showed remarkable improvement with the adoption of the **nested PCR technique** in clinical samples, targeting the C-terminal part of the *hupB* gene, (Fig: 6) and (Table VI- VIII).

EXAMPLES

Bacterial strains: The mycobacterial strains as well as non-mycobacterial strains used in the study have been listed in Table I. In all 80 mycobacterial strains were included in the study besides 10 non-mycobacterial species. Of the 80 mycobacterial isolates included 55 were members of the MTB complex, (*M. tuberculosis* – 25, *M. bovis* – 25, *M. microti*-3, and 1 each of *M. africanum* and *M. canetti*). The details of the *M. bovis* strains included are as follows: 7 from infected cattle housed in the Central Military Veterinary Laboratory, Meerut, India, 9 from National Mycobacterial Repository, JALMA, Agra India, 2 each from Netherlands and Argentina and 3 human isolates from the Netherlands (Drs.J.D.A. van Embden and D.van Soolingen).

Processing of bacilli for specificity analysis

All the mycobacterial and non-mycobacterial strains grown on solid media (LJ slants all mycobacterial species), LB agar (*E. coli*) nutrient agar (*Aspergilus niger*, *Nocardia asteriodes*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*) or blood agar (*Corynebacterium diphtheriae*, *Streptococcus pneumoniae*) were scraped with the help of sterile toothpicks and re-suspended in sterile distilled water containing 0.1% Triton X-100. Re-suspended bacilli were boiled at 100°C for 20 minutes and an aliquot (2µl) was used for PCR.

PCR Analysis:

1) 23S rDNA target: Primers: C, **Seq ID No.9**(5' gtgagcgacgggatttgcctat 3') and L, **Seq ID No. 10**(5' accacccaaaaccggatcgat 3') were used to detect the presence of DNA from organisms belonging to genus Mycobacterium. The expected size of the amplicon was 174bp (Verma et al., 1994; Dasgupta et al., 1998).

2) *hupB* DNA target : Primers **N, Seq ID No. 1** (5' ggagggttgggatgaacaaagcag 3') and **S, Seq ID No. 2** (5' gtatccgtgtgtcttgacctatttg 3') were used to amplify *hupB* gene sequences. The expected size of the amplicon was ~645 bp (Table II, Fig:1) in *M. tuberculosis* and 618 bp in *M. bovis*.

Each reaction (20µl) contained 1.5 mM $MgCl_2$, 0.5 µM of primers, 200 µM dNTPs, 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% Nonidet P40, and 0.5 Units of Taq DNA Polymerase. The PCR reaction was subjected to initial denaturation at 94°C for 10 min., and 35 cycles of each of 1 min at 94°C, 1 min., at 63°C and 1 min at 72°C followed by final extension at 72°C for 30 mins. The fragments were analyzed on a 1.2 % agarose gel and stained with ethidium bromide.

The C-terminal portion of the gene was amplified by using **M, Seq ID No. 3** (5' gcagccaagaaggtagcgaa 3') with **S, Seq ID No. 2** (5' gtatccgtgtgtcttgacctatttg 3'), the expected amplicon was ~ 318 bp.

Nested PCR: DNA extracted from clinical samples / cultivated mycobacteria were processed for PCR with primers Seq.ID. No.1-N and Seq.ID. No.2-S . The PCR product obtained using the primers Seq.ID. No.1-N and Seq.ID. No.2-S was used as target DNA in nested PCR.

Each reaction (40µl) contained 2.5 mM $MgCl_2$, 0.5 µM of primers, 200 µM dNTPs, 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% Nonidet P40, and 0.5 Units of Taq DNA Polymerase. The PCR reaction was subjected to initial denaturation at 95°C for 10 min., and 35 cycles of 1 min at 94°C, 1 min., and 30 seconds at 59°C and final extension at 72°C for 7 mins. The fragments were analyzed on a 3.5 % agarose gel / 8 % non-reducing polyacrylamide gel and stained with ethidium bromide. The C- terminal portion of the gene was also amplified by using Seq.ID. No.4-F (5' ccaagaaggcgacaaagg3') with Seq.ID. No.5-R (5' gacagctttcttggcggg3'), the expected amplicon was ~ 116 bp in case of *M.tuberculosis* and 89 bp in case of *M.bovis*, (Table II, Fig:1).

Southern Hybridization: The PCR amplicons resolved on the agarose gel were transferred on to nitro-cellulose membrane (Southern, 1975). The blots were then hybridized with α-^{32}P labeled 645 bp *hupB* (Seq ID No.6) gene probe from *M. tuberculosis*, (*Pst*I & *Nco*I digest from the plasmid pHLPMT / probe generated by PCR using **N** (Seq ID No.1-N) and **S** (Seq ID No.2-S) primers and *M. tuberculosis*, DNA.).

Restriction Fragment Length Polymorphism:

hupB amplified sequences were digested with HpaII restriction enzyme and the fragments were analyzed on a 12% non-denaturing polyacrlyamide gel. The gel was strained with ethidium bromide and DNA fragments were visualized under ultraviolet light.

DNA Sequencing Analysis: The PCR fragments were sequenced by the Sanger's dideoxy chain termination method (Sanger et al., 1977) using Sequenase Ver 2.0 sequencing kit, α^{35} SdATP and forward/reverse universal M13 primers or internal primers of *hupB*, according to the manufacturer's instructions. The DNA template was alkali denatured and annealed to the primers at -70°C for 1 hour. The GC rich mycobacterial DNA was mixed with 0.5 µg of single strand binding protein prior to labeling. The protein was digested with proteinaseK 0.1 µg at 68°C for 20 mins., following termination of the labeling reaction. The reactions were electrophoresed on a 6% urea –polyacrylamide gel in 1X TBE at 70 W for a suitable time period. The gel was fixed with acetic acid (10%) and methanol (30%) dried and autoradiographed. The PCR fragments obtained in standard strains and isolates were also sequenced commercially by Microsynth, Switzerland.

The specificity of the PCR assay: DNA from 16 mycobacterial and 10 non-mycobacterial species were used as target to establish the specificity of the PCR assay, (Table 1). The DNA extracted from 3 standard strains and 4 clinical isolates of *M. tuberculosis* and *M. bovis* (BCG) were included for amplification using the *hupB* primers (**Seq ID No. 1- N,** and **Seq ID No. 2- S,**, Table II, Fig: 1). Only in case of *M. tuberculosis* H37Rv, H37Ra, *M. bovis* BCG and 5 clinical isolates of *M. tuberculosis* (lanes:1, 2,3,16,17,18 and 20, in Fig:2A and lanes 1 and 12 in Fig.2B) the expected 645 bp fragment was obtained in case of *M. tuberculosis* and 618 bp in case of *M. bovis*. No amplification was seen with *M. microti*, *M. africanum* of the MTB complex, *M. leprae*, MAIS complex and other

mycobacterial species (rapid and slow growers) including *Corynebacterium diphtheriae* and *Nocardia asteriodes* that together make the *Corynebacteria, Nocardia and Mycobacteria* (CNM) group. Amplification was also not seen in other non-mycobacterial species (Fig:2B). The authenticity of the amplified fragment was confirmed by hybridization with α-^{32}P labeled 645 bp fragment (Seq ID No.6) (Fig: 2A' and B'). This confirmed that no other amplification was obtained with any other template DNA that could have been missed by ethidium bromide staining alone. Thus the 5' and 3' primers of *hupB* are specific for *M. tuberculosis* and *M. bovis*.

Sensitivity of *hupB* gene based PCR assay: The sensitivity of DNA PCR amplification (level of detection) was established by adding serial dilutions of mycobacterial DNA (1 ng to 1 fg) in the PCR reaction using primers **Seq ID No. 1- N** and **Seq ID No. 2- S**. It was seen that by ethidium bromide staining alone the detection limit was 50 pg and by hybridization the detection limit increased to 500 fg (Fig: 3A and B). This was equivalent to the detection of 5000 and 50 genome equivalents respectively.

RFLP of PCR Amplicons of *hupB* gene derived from *M. tuberculosis* and *M. bovis*: DNA from different isolates of *M. tuberculosis* and *M. bovis* (listed in Table I) were amplified using **Seq ID No. 1-N** and **Seq ID No. 2- S** primers (645 bp fragment, Table II) and (ii) **Seq ID No. 3- M** (internal primer) and **Seq ID No. 2- S** (318 bp fragment, Fig: 4C, Table II, Fig: 1). PCR amplicons obtained from the DNA of *M. bovis* strains (lanes 4-11, Fig: 4B and 4C) were smaller in size as compared to the PCR amplicons obtained from the *M. tuberculosis* strains (lanes 1-3, Fig:4B and 4C). The results of the PCR assay with the 2 sets of primers have been summarized in Table III.

In order to confirm the difference in 645 and 618 bp PCR fragment sizes, the amplicons were digested with *HpaII* and *HaeIII* (Fig.:4D). The digested fragments were analyzed on 12% non-denaturation polyacrylamide gel. Digestion of 645 bp fragment with *HpaII* clearly revealed that in case of *M. bovis* a ~250 bp (Fig: 4D, lane 3) fragment obtained was smaller in size compared to the ~280 bp bands obtained with *M. tuberculosis* H37Ra & H37Rv (Fig: 4D, lanes 1 and 2). No differences were perceived with *HaeIII* digestion, (Fig: 4D, lanes 5-8). Results, obtained with the amplicon (318 bp) generated in the C – terminal portion of the gene using **Seq ID No. 3- M** and **Seq ID No. 2- S** primers on digestion with *HpaII*, showed similar differences (results not shown) indicating that the PCR-RFLP assay did distinguish between *M. tuberculosis* and *M. bovis* strains.

Sequencing of PCR Amplified Fragment: PCR amplicons obtained from DNA of standard strains of *M. bovis* and *M. tuberculosis* including local isolates of *M. bovis* derived from cattle were sequenced. The PCR amplicons 618 and 645 bp (obtained using Seq ID No. 1-N and 2-S), 318 and 291 bp (obtained using Seq ID No. 3-M and 2-S), 116 and 89 bp (obtained using Seq ID No. 4-F and 5-R) were sequenced to confirm the size differences. Sequence analysis indicated that in *M. bovis* there was a deletion of 27 bp (9 amino acids) in frame after 128th codon in the C terminal part of the gene (Fig: 5,). The histone like gene sequence of *M. bovis* (Accession No.Y18421) and *M. tuberculosis* (Accession No. P95109) has been submitted to the NCBI data base.

Advantages of PCR, RFLP and Nested PCR Assay:

1) Unlike currently available assays the assay provides a method for direct detection and identification of human pathogenic mycobacteria in clinical samples, dairy and meat products. The assay enables the identification

of human pathogenic mycobacteria belonging to the *Mycobacterium tuberculosis* Complex.

2) The method described herein has the unique advantage over existing methods as a means for not only simultaneous detection but also precise identification of two intimately related mycobacteria namely *M. tuberculosis* and *M. bovis*.

3) The PCR, RFLP and nested PCR technique described herein takes advantage of the unique novelty of the *Hup B* gene as a target. The novel primer pairs (Seq. ID. No. 1 & 2; 3 & 2; 4 & 5) designed facilitates the specific amplification of the *Hup B* gene in two known pathogenic mycobacteria namely *M. tuberculosis* and *M. bovis*,

4) The size and sequence difference of the amplified fragments permits their reliable identification, which hitherto was not possible by all other reported methods.

5) More importantly the method described enables the investigator to detect and diagnose dual infection in clinical samples caused by pathogenic mycobacteria other than *M. tuberculosis* such as *M. bovis*.

M.tuberculosis (Human isolates)	H37Rv, H37Ra, Erdman, P8473, P8497, C1207, C1084, 779634, ICC107, ICC120, ICC22, ICC238, ICC136, ICC37, ICC247, ICC16, ICC235, ICC145, ICC06, ICC11, ICC85, ICC95, CSU-17, CSU-27, CSU-20	a,b,c,d,,g n
M. bovis (Cattle Isolates)	T11, AN5, IC378, IC379 , IC380, IC381, IC382, ICC388, ICC391, 117, 126, 73, 130 , CL1, CL3 , CL4 , CL8 , CL10 , CL33 , CL42, Japanese* & Copenhagen*	d,o,p,q
Human Isolates	6 ,47 , 85 ,	o
M. canetti	116	o
M. africanum	81543	e,g
M. microti	OV254, T14,N5	d,f
M. gastri	TMC1456	b
M. chelonae	TMC191,J31	b
M. vaccae	IND123	b
M. avium	NCTC8562, ICC192	d
M intracellulare	TMC1302, N25, N8	d
M. scrofulaceum	TMC1302, MAC29	d
M. gordonae	TMC1324	d
M. fortuitum	5J32, ICC420, ICC419, ICC417, ICC416	g,d,l
M. smegmatis	ATCC27204, LR222, N18	b.d
M. phlei	ND124, N14	b
M. Kansasii	1201	c
M. leprae	Tissue Biopsy	d
M. simae	IN7	d
Corynebacterium diphtheriae	Clinical isolate	h
Streptococcus β- haemolyticus	Clinical isolate	h
Staphylococcus aureus	Clinical isolate	h
Pseudomonas aeruginosa	Clinical isolate	h
Klebsiella pneumoniae	Clinical Isolate	h
Nocardia asteriodes	MTCC274	i
Aspergillus fumigatus	Soil isolate	j
Aspergillus niger	Soil isolate	j
Candida albicans	Clinical isolate	k
Escherichia coli	DH5α, BL21 (DE3)	m

a- P.S. Murthy, UCMS, University of Delhi, India; b- N.K. Jain, NDTC, New Delhi, India; c- C.N. Paramasivan, TRC, Chennai, India; d- V.M. Katoch, JALMA, Agra, India; e- Y.M. Yates, Public Health Laboratory, Dulwich Hospital, London, UK; f- P. Draper, NIMR, Mill Hill, London, UK; g- Kathleen Eisenach, University of Arkansas, USA; h- Dept. of Microbiology,AIIMS, New Delhi, India; i- Microbiological Type Culture Collection, IMTECH, Chandigarh, India; j- Shivkumar, Anna University, Chennai, India; k- Z.U. Khan, V.P. Chest Institute, Delhi, India; l- Jack Crawfort, CDC, Atlanta, GA, USA; m-GIBCO BRL, USA; n-Suman Laal, VA Medical Center, NY U, School of Medicine, New York, USA; o- J.D.A. van Embden, Netherlands; p-Central Military Veterinary Laboratory , Meerut, India; q- Dept. of Paediatrics, AIIMS, New Delhi, India; (*) Human vaccine strain; Numbers in bold - human isolates.

Table II: Primers Used for Amplification of *hupB* Mycobacterial DNA Target

Primer Pair	Sequence of Primer	Target *hup B* gene	Mycobacteria	PCR Product Size
Seq.Id. No.1 - N Seq.Id. No.2 - S	(5'ggagggttgggatgaacaaagcag 3') (5' gtatccgtgtgtcttgacctatttg 3')	Whole gene	*M. tuberculosis*	645 bp
			M. bovis	618 bp
Seq.Id. No.3 - M Seq.Id. No.2 - S	(5' gcagccaagaaggtagcgaa 3') (5' gtatccgtgtgtcttgacctatttg 3').	C terminal	*M. tuberculosis*	318 bp
			M. bovis	291 bp
Seq.Id. No.4 - F Seq.Id. No.5 - R	(5' ccaagaaggcgacaaagg3') (5' gacagctttcttggcggg3').	C terminal	*M. tuberculosis*	116 bp
			M. bovis	89 bp

Sequence

<213> OrganismName : Mycobacteria
<400> PreSequenceString :
VKPTSVPAFR PGAQFK 16
<212> Type : PRT
<211> Length : 16
SequenceName : seq id no. 6 (Change to 8)
SequenceDescription :

Sequence

<213> OrganismName : hup B - M. tuberculosis, Rv2986c, Accession No. P95109
<400> PreSequenceString :
atgaacaaag cagagctcat tgacgtgctc acacagaaat tgggctcgga ccgtcggcag 60
gcgaccgccg ccgtcgagaa tgtcgttgac acgattgtgc gtgcggtaca caaaggcgac 120
agcgtcacca ttaccgggtt cggtgtgttc gaacagcgtc gccgcgcggc tcgagtggcc 180
cgcaatccgc gtaccggcga gacagtaaag gtgaagccga cgtcggtgcc ggcgttccgc 240
ccgggcgcgc aattcaaagc ggttgtgtct ggcgcgcagc gtctcccggc agaaggaccc 300
gctgttaagc gtggtgtggg ggccagtgca gccaagaagg tagcgaagaa ggcacctgcc 360
aagaaggcga caaaggccgc caagaaggcg gcgaccaagg cgcccgccag gaaggcggcg 420
accaaggcgc ccgccaagaa agcggcgacc aaggcgcccg ccaagaaagc tgtcaaggcc 480
acgaagtcac ccgccaagaa ggtgaccaag gcggtgaaga agaccgcggt caaggcatcg 540
gtgcgtaagg cggcgaccaa ggcgccggca aagaaggcag cggccaagcg gccggctacc 600
aaggctcccg ccaagaaggc aaccgctcgg cgggtcgca aatag 645
<212> Type : DNA
<211> Length : 645
SequenceName : Seq id no.7 (Change to 6)
SequenceDescription :

Sequence

<213> OrganismName : Hlp of Mycobacterium bovis, Acession No. Y18421
<400> PreSequenceString :
atgaacaaag cagagctcat tgacgtgctc acacagaaat tgggctcgga ccgtcggcag 60
gcgaccgccg ccgtcgagaa tgtcgttgac acgattgtgc gtgcggtaca caaaggcgac 120
agcgtcacca ttaccgggtt cggtgtgttc gaacagcgtc gccgcgcggc tcgagtggcc 180
cgcaatccgc gtaccggcga gacagtaaag gtgaagccga cgtcggtgcc ggcgttccgc 240
ccgggcgcgc aattcaaagc ggttgtgtct ggcgcgcagc gtctcccggc agaaggaccc 300
gctgttaagc gtggtgtggg ggccagtgca gccaagaagg tagcgaagaa ggcacctgcc 360
aagaaggcga caaaggccgc caagaaggcg gcgaccaagg cgcccgccaa gaaagcggcg 420
accaaggcgc ccgccaagaa agctgtcaag gccacgaagt cacccgccaa gaaggtgacc 480
aaggcggtga agaagaccgc ggtcaaggca tcggtgcgta aggcggcgac caaggcgccg 540
gcaaagaagg cagcggccaa gcggccggct accaaggctc ccgccaagaa ggcaaccgct 600
cggcggggtc gcaaatag 618
<212> Type : DNA
<211> Length : 618
SequenceName : Seq id no.8 (Change to 7)
SequenceDescription :

Sequence

<213> OrganismName : 23S- Genus Mycoabcteria-C
<400> PreSequenceString :
gtgagcgacg ggatttgcct at 22
<212> Type : DNA .
<211> Length : 22
SequenceName : seq id no. 9
SequenceDescription :

Sequence

<213> OrganismName : 23S-Genus Mycobacteria-L
<400> PreSequenceString :
accacccaaa accggatcga t 21
<212> Type : DNA
<211> Length : 21
SequenceName : Seq id no. 10
SequenceDescription :

Table III A: Representative results of *hupB* PCR Assay with Strains of *M.tuberculosis*

M.tuberculosis		
Strain	**Source**	**PCR Amplified Product Obtained**
		645 / 318 bp
H37Rv	ATCC[a]	+ / +
H37Ra	ATCC	+ / +
Erdman	ATCC	+ / +
779634	Human isolate[b]	+ / +
P8473	Human Isolate	+ / +
P8497	Human Isolate	+ / +
C1207	Human isolate	+ / +
C1084	Human isolate	+ / +

a- Dr.Kathleen Eisenach, University of Arkansas, USA

b- Dr. C.N.Paramasivan, Tuberculosis Research Centre, Chennai, India

Table III B: Representative results of *hupB* PCR Assay with Strains of *M. bovis*

M. bovis		
Strain	**Source**	**PCR Amplified Product Obtained**
		618 / 291 bp
AN5	Cattle isolate [c]	+ /+
IC378	Cattle isolate	+ /+
IC379	Cattle isolate	+ /+
IC380	Cattle isolate	+ /+
IC381	Cattle isolate	+ /+
IC382	Cattle isolate	+ /+
117	Cattle isolate (Argentina)[d]	+ /+
126	Cattle isolate (Argentina)	+ /+
BCG	Japanese [e]	+ /+
BCG	Copenhagen	+ /+

c- Dr. V.M.Katoch, JALMA, Agra, India; d- Dr. J.D.A. van Embden, Netherlands

e- Pediatrics Dept. of AIIMS, New Delhi.

Table IV: Results of the Direct PCR assay carried out with Bovine Samples

Samples	*Detection of Mtb Complex by the PCR Assay*		
	Number Tested	*Number Positive*	*Percent*
Lymph Gland Biopsy	89	21	23.6
Blood (Heparinsed)	89	01	01.1
Pharyngeal Swab	89	02	02.2
Faeces	89	02	02.2
Rectal Pinch	89	03	03.4
Milk	89	11	12.4
Total Tested	534	40	07.5

The following bovine samples were found to be appropriate for the PCR based assay for detection of bovine tuberculosis: Lymph Gland Biopsy and Milk were found to be the best (Chi square test, p value < 0.05 at significance level, (SAS 8.0, Statistical Software).

Table V : Comparative Analysis Of Clinical & AFB Status of Cattle With Direct PCR Results

Clinical Status		*Number Positive For*	
Category	*Number*	*Acid Fast Bacilli*	*PCR*
A	17	13	07
B	12	Nil	Nil
C	20	12	09
D	20	05	08
E	20	Nil	02
Total	89	30 (33.7%)	26 (29.2%)

A - Tuberculin Positive with Clinical Signs of Tuberculosis
B - Tuberculin Positive, Apparently healthy Animal
C - Tuberculin Negative with Clinical Signs of Tuberculosis
D - Tuberculin Negative Apparently healthy Animal
E - Animal Infected with non-mycobacterial infection

Among the clinical categories of animals investigated, bovine tuberculosis was detected least in animals infected with non-mycobacterial micro-organisms (Category E), compared to all other categories ($p<0.05$, (Chi square test, p value < 0.05 at significance level, SAS 8.0, Statistical Software).

Table VI: Nested PCR based Identification of Pathogenic Mycobacteria in Cattle Derived Samples.

Samples[a]	N- PCR based Identification of[b]	
	M.tuberculosis	*M.bovis*
Lymph Gland Biopsy	15	18
Blood[c]	14	14
Milk	26	26
Total Tested 192	55 (28.6%)	58 (30.2%)

a- 64 Samples tested in each category
b- Nested PCR for the C terminal region of the *hup B* gene
c- Citrated Blood

Table VII : Comparative Analysis Of Clinical & AFB Status of Cattle With Nested PCR Results

Clinical Status		*Number Positive For*	
Category	*Number*	*Acid Fast Bacilli*	*N-PCR*
A	20	09	19
B	17	03	16
C	09	03	08
D	10	Nil	07
E	08	Nil	07
Total	64	15 (23.4%)	57 (89.0%)

A - Tuberculin Positive with Clinical Signs of Tuberculosis
B - Tuberculin Positive, Apparently healthy Animal
C - Tuberculin Negative with Clinical Signs of Tuberculosis
D - Tuberculin Negative Apparently healthy Animal
E - Animal Infected with non-mycobacterial infection

Table VIII: Comparison of Bacteriological and PCR-RFLP / Nested - PCR based identification of mycobacterial isolates derived from cattle

Identification of Cattle Derived Mycobacterial Isolates			
Isolate	Classical Criteria	PCR Based Identification	
		M.tuberculosis	*M.bovis*
173	*M.bovis*	+	–
315	*M.tuberculosis*	+	–
262	*M.bovis*	–	+
95	*M.bovis*	–	+
101	*M.bovis*	+	–
113	*M.bovis*	–	+
155	*M.bovis*	–	+
28	*M.bovis*	–	+
36	*M.bovis*	+	–
33	*M.bovis*	–	+